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DOI: https://doi.org/10.1159/000345362

Original Paper

Caries Research

Caries Res 2013;47:162–170
DOI: 10.1159/000345362

Received: August 20, 2012
Accepted after revision: October 22, 2012
Published online: December 7, 2012

Different Protocols to Produce Artificial Dentine Carious Lesions in vitro and in situ: Hardness and Mineral Content Correlation

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Key Words
Demineralization · Dental caries · Dentine · Hardness · Mineral content

Abstract
This study compared dentine demineralization induced by in vitro and in situ models, and correlated dentine surface hardness (SH), cross-sectional hardness (CSH) and mineral content by transverse microradiography (TMR). Bovine dentine specimens (n = 15/group) were demineralized in vitro with the following: MC gel (6% carboxymethylcellulose gel and 0.1 M lactic acid, pH 5.0, 14 days); buffer I (0.05 M acetic acid solution with calcium, phosphate and fluoride, pH 4.5, 7 days); buffer II (0.05 M acetic acid solution with calcium and phosphate, pH 5.0, 7 days), and TEMDP (0.05 M lactic acid with calcium, phosphate and tetraethyl methyl diphosphonate, pH 5.0, 7 days). In an in situ study, 11 volunteers wore palatal appliances containing 2 bovine dentine specimens, protected with a plastic mesh to allow biofilm development. The volunteers dripped a 20% sucrose solution on each specimen 4 times a day for 14 days. In vitro and in situ lesions were analyzed using TMR and statistically compared by ANOVA. TMR and CSH/SH were submitted to regression and correlation analysis ($p < 0.05$). The in situ model produced a deep lesion with a high R value, but with a thin surface layer. Regarding the in vitro models, MC gel produced only a shallow lesion, while buffers I and II as well as TEMDP induced a pronounced subsurface lesion with deep demineralization. The relationship between CSH and TMR was weak and not linear. The artificial dentine carious lesions induced by the different models differed significantly, which in turn might influence further de- and remineralization processes. Hardness analysis should not be interpreted with respect to dentine mineral loss.

The induction of artificial carious lesions in bovine dentine is an important tool to investigate strategies for the prevention or treatment of dentine carious lesions [Okuyama et al., 2006; Zaura et al., 2007; Preston et al., 2008; Pavan et al., 2011], which is a common oral problem for patients suffering from periodontal recession [Ravald and Starkhammar Johansson, 2012].

In vitro models are particularly well suited to experiments whose objective is to test a single process in isolation, where a more complex situation with many vari-

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ables may confound the data. The composition of the various demineralizing systems (gels and solutions) has been developed in an attempt to simulate the conditions of cariogenic biofilm during sugar metabolism. However, it must be kept in mind that the concentrations of calcium and phosphate, and in some cases the pH values, chosen in vitro are lower than in the natural intraoral situation in order to induce a faster demineralization than occurs in vivo. Therefore, differences among these solutions or gels, such as initial degree of saturation with respect to tooth minerals, fluoride concentration, kind of acid and viscosity can result in remarkable differences in physical and mechanical characteristics of the demineralized substrate, such as mineral distribution characteristics [Arends et al., 1987; McIntyre et al., 2000], chemical composition [Lynch and ten Cate, 2006] and hardness [Magalhães et al., 2009; Marquezan et al., 2009].

On the other hand, in situ protocols for the development of carious lesions are closer to the clinical situation due to the presence of dental biofilm and the exposure to sucrose [Ögaard and Rölla, 1992]. However, to speed the demineralization process, the cariogenic challenges applied in most in situ studies are also more aggressive than those that normally occur during the development of natural carious lesions [Hara et al., 2003; Aires et al., 2008].

Despite the large diversity of studies using different protocols to induce dentine carious lesions [McIntyre et al., 2000; Buchalla et al., 2003; Zaura et al., 2007; Marquezan et al., 2009], there is no study comparing in vitro to in situ models with respect to their potential to induce demineralization. It is important to point out that the kind of lesion influences the behavior to further de- or remineralization, as the surface layer, porosity and lesion depth can play an important role in the mineral diffusion [ten Cate, 1994; Kawasaki et al., 2000; Preston et al., 2008; Bertassoni et al., 2010].

Depth-related properties of artificial caries lesions can be described by mineral content and hardness profiles. Transverse microradiography (TMR) provides a quantitative measure of the mineral content and has been widely used also to assess transverse mineral distribution of caries lesions in dentine [Inaba et al., 1997; Buchalla et al., 2003]. Therefore, this method is considered as the gold standard for the quantification of the mineral content of caries lesions in vitro. On the other hand, cross-sectional hardness (CSH) reflects the mechanical resilience of the dental hard tissue. However, it is debated whether surface hardness (SH) or CSH analysis might reflect depth mineral alterations of carious dental tissues or if it is able to detect differences among the lesions provoked by different protocols [Buchalla et al., 2008; Magalhães et al., 2009].

A previous work of our group has shown that CSH, as an alternative to TMR, is not a valid surrogate for mineral content of demineralized enamel [Magalhães et al., 2009]. Dentine caries is a diffusion-controlled process. The demineralization involves not only chemical dissolution of the inorganic material, but also the exposure and degradation of the organic matrix, mainly collagen type I [van Strijp et al., 2003; Chaussain-Miller et al., 2006]. Thus, it is expected that the high organic content of dentine could influence the measurement of mechanical properties [Herkströter et al., 1989; Balooch et al., 2008]. However, there is no study testing the correlation between hardness and mineral content of dentine demineralized by different laboratory protocols so far.

Therefore, the present study aimed (1) to compare different in vitro and in situ models proposed in the literature to induce artificial carious lesions in dentine and (2) to correlate the data of SH and CSH with mineral content profiles using TMR.

Materials and Methods

Ethical Aspects

This study was approved by the local ethical research committee (FOB-USP, process No. 057/2009). For the in situ experiment, 11 adult volunteers took part after signing an informed consent. They fulfilled the inclusion criteria (physiological salivary flow rates: stimulated: >1 ml/min, nonstimulated: >0.25 ml/min; good oral health: no frank cavities or significant gingivitis/periodontitis) without violating the exclusion criteria (systemic illness, pregnancy or breastfeeding, use of fixed or removable orthodontic appliances, use of fluoride mouth rinse or professional fluoride application in the last 2 months, hyposalivation).

Specimen Preparation

Root dentine specimens (4 × 4 × 3 mm) were prepared from bovine incisors, which were freshly extracted and stored in water containing NaCl (0.9%) and thymol (0.1%) until used. The teeth were cut using an IsoMet Low Speed Saw (Buehler Ltd., Lake Bluff, Ill., USA) and two diamond disks (Extec Corp., Enfield, Conn., USA), which were separated by a 4-mm-wide spacer. The dentine surface of the samples was ground flat using water-cooled silicon carbide disks (320-, 600- and 1,200-grade papers, ANSI grit; Buehler, Lake Bluff, Ill., USA), and polished using felt paper wet with diamond solution (1 μm; Buehler), resulting in the removal of about 200 μm of the outer cement/dentine. This was controlled with a micrometer.

Prior to the experiment, the specimens were disinfected by dipping in 70% alcohol solution for 30 min in addition to the previous immersion in thymol solution [Schlueter et al., 2009]. Two thirds of the surface was covered with nail varnish in order to cre-

ate control areas to both sides of a central band of exposed dentine (approx. 1–1.5 mm).

For the in vitro experiments, each n = 15 specimens were randomly allocated to each of the four groups. For the in situ experiment, n = 22 specimens were randomly allocated to 11 subjects (n = 2/subject). The randomization was done according to SH means (29 ± 6 KHN/group or subject). SH determination is described below.

In situ Experiment

Acrylic palatal appliances were made for each of 11 subjects with two positions for the specimens. In order to protect the dentine surface from mechanical disturbance and allow plaque accumulation, a plastic mesh with 1 × 1 mm apertures (Sanremo, Brazil) was fixed over the cavities containing the specimens, leaving a 1-mm space between mesh and specimen surface.

During 14 days, the appliances were only removed for the main meals (4 times a day, maximum 1 h each, interval between meals 2–3 h) and for the application of the sucrose solution (20% weight/volume, 1 drop/specimen) 4 times a day (each 5 min) [Hara et al., 2003; Aires et al., 2008]. Thereafter, the appliance was replaced into the mouth. The sucrose solution was renewed every 3 days of the experiment.

Seven days prior to and throughout the in situ phase, the subjects brushed their teeth with fluoride-free toothpaste in order to avoid any residual effect of fluoride sources on the specimens. The specimens were not brushed to allow for plaque accumulation.

In vitro Experiment

In the MC gel group, the specimens were covered with 0.5 cm 6% carboxymethylcellulose gel that was left to set overnight at 4°C in vials of 10 ml. Therefore, they were covered with an equal volume (1.5 ml) of 0.1 M lactic acid, pH adjusted to 5.0, and incubated for 14 days [Inaba et al., 1997]. In the buffer I group, the specimens were immersed in 30 ml of 50 mM acetate buffer solution containing 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4 and 0.5 ppm F, at pH 4.5, for 7 days [ten Cate and Duijsters, 1983; McIntyre et al., 2000]. In the buffer II group, the specimens were immersed in 30 ml of 50 mM acetate buffer solution containing 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, at pH 5.0, for 7 days [ten Cate and Duijsters, 1982; Damen et al., 1998]. In the TEMDP group, the specimens were immersed in 30 ml of 50 mM lactate buffer containing 3 mM $CaCl_2$, 3 mM KH_2PO_4, 6 μM tetraethyl methyl diphosphonate and traces of thymol, at pH 5.0, for 7 days [Buskes et al., 1985; Buchalla et al., 2003]. In all in vitro models, the specimens were separately immersed in unstirred solutions or gel at 37°C. Table 1 summarizes the degrees of saturation with respect to dentine minerals, pH and exposure time. The degree of saturation was calculated using a software program [Shellis, 1988].

The specimens were immersed in deionized water to avoid shrinkage of the dentine before and after the experiment.

Hardness Measurement

Dentine SH was measured using a microhardness tester (HMV-2; Shimadzu Corporation, Tokyo, Japan) and a Knoop diamond, with a load of 10 g applied for 10 s. Five indentations, 100 μm apart, were made in the center of the dentine specimens at baseline (SH_0) and at the end of the experiment (SH_1).

To perform CSH tests, the specimens were sectioned once with a diamond band saw, perpendicularly to the surface and the protected areas through the center. One half of each sample was embedded in acrylic resin and polished as described before, while the other half was prepared further for TMR analysis. The specimens were maintained in deionized water until the analysis. For CSH determination the water was removed from the surface using a paper, and three rows of 7 indentations each were made, one in the central region of the exposed area and the other two at 100 μm distance to both sides of the central row, using a 10 g load for 10 s. The indentations were made at 10, 30, 50, 70, 90, 110 and 220 μm from the outer dentine surface. The mean values of all 3 measuring points at each distance from the surface were averaged (kgf/mm^2).

Table 1. Initial degree of saturation, pH and exposure time in each protocol in vitro at 37°C with P_{CO_2} = 0 atm

Protocol	HAP	OCP	DCPD	FAP	pH	Exposure time, days
MC gel	–	–	–	–	5.0	14
Buffer I	0.30	0.13	0.13	1.51	4.5	7
Buffer II	0.66	0.25	0.27	–	5.0	7
TEMDP	0.72	0.27	0.24	–	5.0	7

MC gel is infinitely undersaturated with respect to all calcium phosphates [Shellis, 1988]. HAP = Hydroxyapatite; OCP = octacalcium phosphate; DCPD = dicalcium phosphate dehydrate; FAP = fluorapatite.

Transverse Microradiography

The other half of the specimens was additionally cut and hand polished plane-parallel from both cut sides with water-cooled silicon carbide disks (320-, 600-, and 1,200-grade papers, ANSI grit; Buehler) to a thickness of 138 ± 7.6 μm. After the immersion of the specimens in ethylene glycol (Sigma-Aldrich, Steinheim, Germany) for 24 h in order to avoid shrinkage during X-ray exposure due to desiccation [Buchalla et al., 2003], micrographs of each section together with an aluminum calibration step wedge with 14 steps were taken. High-speed holographic film (SO 253; Kodak AG, Stuttgart, Germany) was exposed with Ni-filtered quasi-monochromatic Cu Kα X-rays (λ = 0.154 nm) from a 1 × 10 mm focus X-ray tube (PW2233/20; Philips, Kassel, Germany) at 20 kV and 20 mA (PW 3830 generator; Philips) for 15 s. The film-focus distance was 40 cm. The developed film was analyzed using a transmitted light microscope with ×20 objective (Axioplan; Zeiss, Oberkochen, Germany) equipped with a CCD camera (XC-77CE; Sony, Tokyo, Japan) and a PC with frame grabber, data acquisition and calculation software (TMR 1.25e; Inspektor Research BV, Amsterdam, The Netherlands). One measurement was done for each microradiogram in the center of the demineralized window. Thereby, a field of 350 × 400 μm was analyzed by averaging the gray value of pixel columns parallel to the outer surface of the specimen. The horizontal resolution initially was 2 μm.

The mineral content was calculated assuming that the mineral content of sound dentine is 50 vol% [Buchalla et al., 2003]. The lesion depth was calculated using a threshold of 95% of the mineral content of sound dentine (i.e. 47.5%). Integrated mineral loss (ΔZ),

Table 2. Quadratic and linear regression of CSH or √CSH and mineral content for the different models and for all models combined

Analysis	Parameter	MC gel	Buffer I	Buffer II	TEMDP	In situ	Total
Quadratic		√CSH	CSH	CSH	CSH	CSH	√CSH
	Intercept	0.02	20.49	15.63	9.69	2.14	1.16
	Linear slope	0.08	–0.55	0.08	0.31	0.63	0.16
	Quadratic slope	0.0001	0.0124	0.0014	–0.0005	–0.0060	–0.0021
	Adjusted r^2	0.39	0.054	0.07	0.23	0.46	0.33
Linear		√CSH	CSH	CSH	CSH	CSH	√CSH
	Intercept	–0.07	15.00	14.51	10.22	3.09	2.05
	Slope	0.09	0.06	0.16	0.27	0.35	0.06
	r^2	0.39	0.01	0.07	0.23	0.45	0.26

The table shows the relation between mineral content and hardness at a depth of 10, 30, 50, 70, 90, 110 and 220 μm (X variables indicated, CSH or √CSH, are those that gave the highest r^2 value). $p < 0.05$ for all regression analyses.

the average mineral loss over the lesion depth (R), the mean thickness of the 'pseudo-intact' surface layer (SL) and the maximum mineral content of the surface layer (Z_{max}) were also calculated.

Statistical Analysis

Means and standard deviations were calculated for SH, CSH and TMR parameters (ΔZ, lesion depth, SL, Z_{max} and R). Equality of variances and normal distribution of the data were tested for all the variables using the Bartlett and Kolmogorov-Smirnov tests, respectively (GraphPad Instat for Windows version 4.0, San Diego, Calif., USA).

To analyze a possible relationship between CSH and mineral content, the data (CSH and mineral content) for each lesion type at a depth of 10, 30, 50, 70, 90, 110 and 220 μm and the combined data from all lesions were submitted first to quadratic regression and then to linear regression (Statistica; Statsoft, Tulsa, Okla., USA). Mineral content was regressed on both hardness and on its square root [Featherstone et al., 1983; Kielbassa et al., 1999]; in this case, the highest R values using hardness or its square root were presented. The correlations between SH_1, $\sqrt{SH_1}$, %SHC and surface layer thickness (SL), maximum mineral content of the surface layer (Z_{max}), lesion depth, integrated mineral loss (ΔZ) and average mineral loss (R) were also examined (Pearson's coefficient).

For comparison among the protocols, the data (Z_{max}, lesion depth, SL and ΔZ) passed the normality test, but the variances were not homogeneous. Therefore, these data were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test. The R values were compared by ordinary ANOVA followed by Tukey's test (GraphPad Instat for Windows version 4.0).

The level of significance for all tests was set at 5% (n = 15 specimens).

Results

All 11 subjects included in this study were able to finish the in situ phase, but some specimens got lost. Thus, only 18 specimens from the in situ experiment could be analyzed. In the in vitro experiment, CSH of 3 (buffer I) and 4 specimens (MC gel) could not be measured due to softening.

Relationships between Hardness and Mineral Content

The quadratic and linear regression showed a weak relation between CSH or √CSH and mineral content for each group and for all groups together (table 2; $p < 0.05$). Generally, the coefficient of mineral content determination from hardness values was lower than 0.50. The same findings were shown when SH_1, $\sqrt{SH_1}$ and %SHC were correlated to TMR parameters; there was a low correlation between the variables, and most of them were not statistically significant. The only significant correlations were found for buffer II ($SH_1 \times Z_{max}$, $r = 0.62$, $p = 0.01$) and TEMDP ($SH_1 \times \Delta Z$, $r = -0.70$, $p = 0.004$). Therefore, the other correlations ($p > 0.05$) were not presented in the Results section because they were not statistically significant.

Differences among Types of Lesion

As the hardness showed no relation with the mineral content, the lesions were compared only using the TMR parameters.

Generally, the in situ model produced an intermediate lesion depth and integrated mineral loss (ΔZ), with the highest R value. The MC gel produced the shallowest and the least demineralized lesion. The buffers I and II as well as TEMDP induced a subsurface and deep dentine demineralization. Buffer I additionally produced the deepest lesion with the highest integrated mineral loss (ΔZ) compared to the other groups. Table 3 shows an overview of all TMR parameters.

Table 3. Summary and statistical comparisons for all TMR parameters (mean ± SD)

	MC gel	Buffer I	Buffer II	TEMDP	In situ
Surface layer thickness, μm	3 ± 4^{a}	14 ± 10^{b}	14 ± 7^{b}	12 ± 8^{b}	1 ± 3^{a}
Maximum surface layer mineral content, vol%	6 ± 9^{a}	31 ± 22^{b}	37 ± 7^{b}	44 ± 7^{b}	2 ± 5^{a}
Lesion depth, μm	87 ± 20^{a}	262 ± 25^{c}	163 ± 30^{b}	163 ± 30^{b}	$137 \pm 49^{a, b}$
Integrated mineral loss, vol% μm	$1{,}709 \pm 301^{a}$	$7{,}070 \pm 1{,}071^{d}$	$3{,}065 \pm 772^{b, c}$	$2{,}279 \pm 591^{a, b}$	$4{,}406 \pm 1{,}973^{c, d}$
Average mineral loss over the lesion depth, vol%	20 ± 4^{b}	27 ± 4^{c}	19 ± 4^{b}	14 ± 3^{a}	31 ± 5^{d}

n = 15/group for the in vitro models and n = 18 for the in situ model. Different superscript letters in the same line show significant differences among the models (ANOVA for R values and Kruskal-Wallis for the other parameters, $p < 0.0001$).

In respect to the surface layer (SL and Z_{max}), only buffer II and TEMDP produced a well-developed surface layer. For the in situ model and the in vitro models, MC gel and buffer I, the surface layer was visible only in 2, 6 and 11 specimens, respectively. Figure 1 shows a representative image and mineral content profile for each group.

Discussion

In the present study, poor linear regression between CSH or square root of CSH and mineral content could be detected considering the data from each single model and the models overall. This finding is in accordance with previous studies focusing on enamel [Buchalla et al., 2008; Magalhães et al., 2009].

Furthermore, there was also only a low or even no correlation between the SH and some TMR parameters. The statistical relationship between both methods was very weak for dentine compared to previous results from a similar study performed in enamel [Magalhães et al., 2009]. This was expected as the high organic content, and thus the elastic properties of the dentine [Herkströter et al., 1989], influences the hardness measurement. According to Marshall et al. [2001], the mechanical properties of dentine measured under hydrated conditions – as done in the present study – provides a more realistic estimation of the in vivo situation. Hardness even in sound dentine is not evenly distributed. The peritubular dentine is harder than the intertubular areas [Kinney et al., 1996], which cannot be distinguished using microhardness testing. If hardness differences within the micrometer range are in focus, nanohardness testing is required [Bertassoni et al., 2011].

As previously discussed, the variability of hardness (SH and CSH) data is high compared to the mineral content, which may be partly attributed to the different volumes that are 'probed' by the indenter compared to the resolution of the X-ray. The hardness measurement at each first depth (especially at 10 and 30 μm depth) of the demineralized surface is not reliable due to the size of the indentation and because the edge of the specimen is very close to the indentation. Therefore, and because of the limited resolution, the exact depth of the lesion is also difficult to identify using hardness indentations 20 μm apart. Furthermore, in the case of the dentine, the relationship between the organic component and mineral, and the degree of humidity are factors that influence the mechanical testing. CSH of some samples in two models (buffer I and MC gel) could be not measured using Knoop indentation due to the high level of softening. On the other hand, TMR measures the mineral content at a much higher resolution (in this study every 2 μm depth). Its accuracy has some limitations only at the outermost 10 μm of the specimen [Magalhães et al., 2009].

Although the CSH gives important evidence regarding the mechanical resilience of the demineralized enamel [Magalhães et al., 2009], it cannot be used to estimate mineral content reliably, particularly in the case of the dentine. The same is valid for SH measurement, which showed only few significant correlations with TMR parameters in the models buffer II and TEMDP. This finding pointed out that the relationship between SH and mineral content might also depend on the type of lesion, not being applicable in all cases.

Regarding the different models to prepare artificial caries lesions, buffer I generally showed higher subsurface mineral loss and lesion depth than the other models. It is important to keep in mind that the demineralization is determined by many factors such as the pH (pH 4.5–5.0), which influences predominantly the rate of demineralization and consequently the time of the experiment

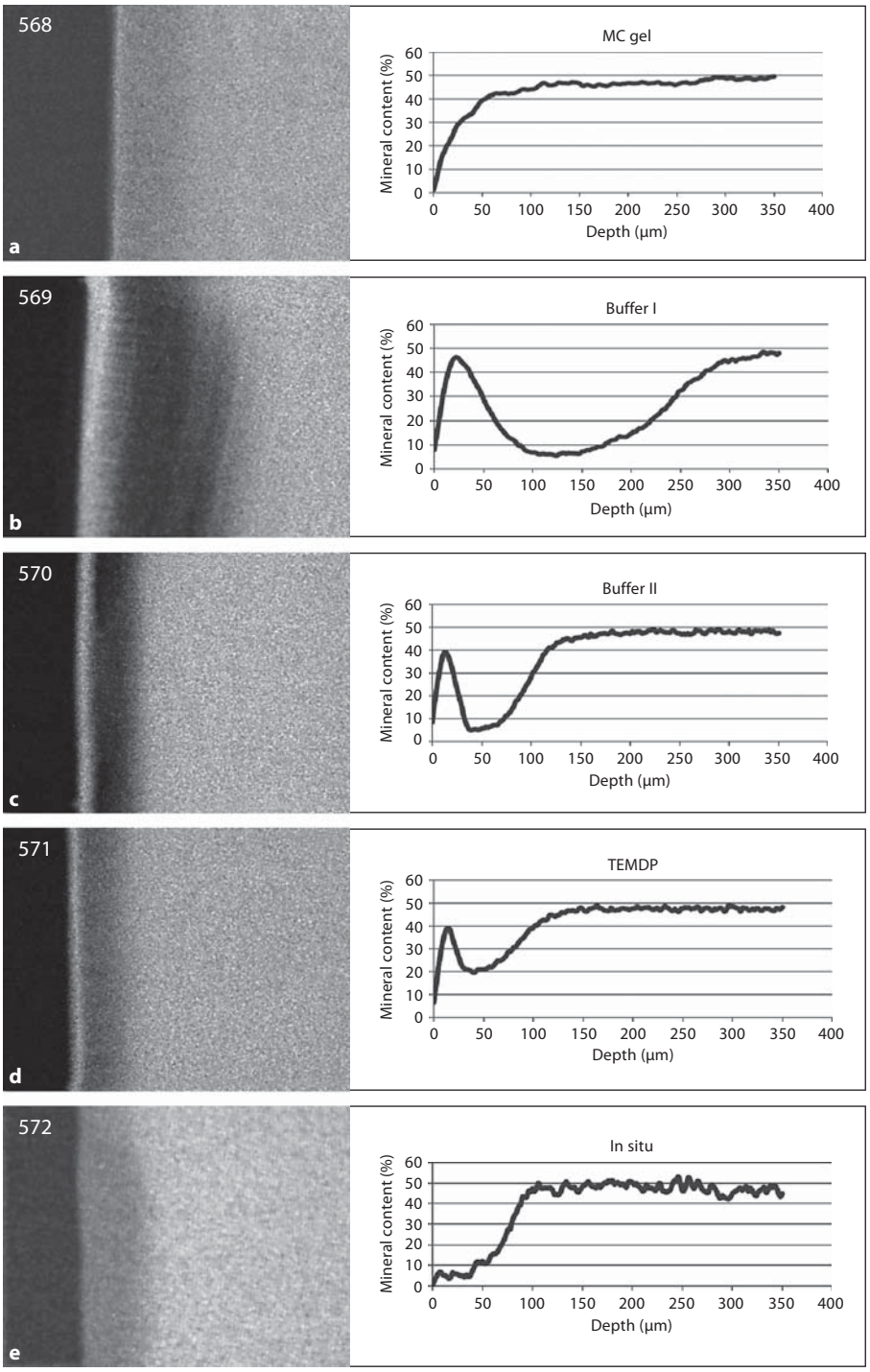

Fig. 1. TMR image and mineral content profile of a representative specimen from each model. **a** MC gel. **b** Buffer I. **c** Buffer II. **d** TEMDP. **e** In situ.

[Theuns et al., 1984b], as well as the content of undissociated acid concentration, degree of saturation, presence of dissolution inhibitors (fluoride, phosphate and some proteins) and temperature [Arends and Christoffersen, 1986; Amaechi et al., 1998]. In the case of buffer I lesions, the results might be explained by the lower degree of saturation regarding hydroxyapatite, octacalcium phosphate and dicalcium phosphate dihydrate compared to the other models (table 1). Buffer I was saturated with respect to fluorapatite, which might have influence on the forma-

tion of the pseudo-intact surface layer evident in most of the specimens from this group. According to Damen et al. [1998], the addition of fluoride to demineralizing solutions does not affect the lesion depth, but the preservation of a mineralized surface layer. The surface layer can also be formed by the reprecipitation of the minerals from the advancing front of the lesion into the surface [Phanksol et al., 1985]. Despite the presence of fluoride, only 11 specimens from buffer I presented a surface layer, showing that the presence of fluoride did not automatically ensure the development of a surface layer under these severely demineralizing conditions.

Buffer II and TEMDP also produced a deep subsurface lesion with similar depth and integrated mineral loss. However, TEMDP produced a highly mineralized surface layer and the mineral loss over the depth was lower compared to all groups, which might be explained by the presence of tetraethyl methyl diphosphonate, a dissolution inhibitor [Buskes et al., 1985; Arends and ten Bosch, 1992].

The preservation of the surface layer is influenced by many factors, such as the presence of calcium and phosphate [Groot et al., 1986], fluoride in liquid phase [Theuns et al., 1984c; Arends and Christoffersen, 1986; Damen et al., 1998] and the time after an initial demineralization [Theuns et al., 1983]. Dentine caries lesions initially do not show a surface layer as was the case in the in situ model; the surface layer is formed over time and its thickness, once formed, appears to be roughly constant [Theuns et al., 1984a, c; Arends and Christoffersen, 1986].

Although the in situ model also produced a deep lesion with the highest mineral loss (high R value), the surface layer was not evident in most samples (only 2 specimens exhibited a surface layer). It can be speculated that the low level of fluoride in the oral environment could be responsible for this finding associated with the severe cariogenic challenge in a short time period. Another possibility is the degradation of the demineralized organic matrix by collagenases from the host or microorganisms, impairing the formation of the surface layer and enhancing the demineralization [Ketler et al., 1994; Tjäderhane et al., 1998; van Strijp et al., 2003]. This hypothesis was previously discussed by Marquezan et al. [2009]. The authors inferred that the lesions produced in vitro simulate the caries-affected dentine, while in the presence of microorganism (as is expected in an in situ model) a lesion might be created similar to caries-infected dentine. An interesting finding of our study was that the in situ protocol presented the highest r^2 value in the regression analysis. It might be speculated that the degradation of the demineralized organic matrix, to the same extent, could reduce the influence of collagen properties on the hardness measurement, improving the relationship between hardness and mineral content.

MC gel produced the shallowest lesion in accordance with a previous study performed in enamel [Magalhães et al., 2009]. The mineral saturation might be reached with time (MC gel presented the longest exposure time), depending on the volume (MC gel presented the lowest volume) and the viscosity of demineralization solution/gel relative to the area of tooth exposed to demineralizing solution/gel. Accordingly, in the case of MC gel, some reduction in calcium activity might have occurred [Lynch et al., 2006] due to the calcium-binding activity of methylcellulose. The MC gel method is the only in vitro method tested in this study that employs a diffusion barrier on top of the dentine surface, similar to dental plaque. Due to the gel consistency, diffusion processes are slowed down markedly compared to buffer solutions.

Generally, our results are in agreement with other studies [McIntyre et al., 2000; Marquezan et al., 2009], in which the demineralization was highest for buffers followed by TEMDP and MC gel. Considering the formation of a subsurface lesion and the results of the present study, buffer II and TEMDP should be appropriate models to be recommended for the laboratory preparation of dentine carious lesions. Generally, both models produced homogeneous and deep lesions, in which a surface layer could be seen in all specimens. Furthermore, both methods are reliable and simple to perform.

The different physical and mechanical properties of the lesions produced by these five models might influence the results of subsequent demineralization and remineralization (such as saliva and fluoride) protocols [Mukai and ten Cate, 2002]. Therefore, further studies are needed to prove if the differences found in properties of the lesions might influence the results of demineralization and or remineralization protocols in vitro and in situ.

Future studies should also analyze which kind of lesion created by the present models behaves most similarly to natural lesions [Marquezan et al., 2009]. It has to be taken into consideration that the in vitro lesions are unable to simulate biological events such as bacterial penetration, collagen degradation, tubular occlusion and reactionary dentine [Shellis, 1994; Marquezan et al., 2009]. Also, a point of interest is whether bovine dentine is an appropriate substitute for human dentine. In this respect, some studies have shown similarity in the mineral loss

and lesion depth between both substrates when they were subjected to demineralization [Mellberg, 1992; Hara et al., 2003].

Therefore, from the results of the present study it can be concluded that: (1) the models for producing artificial dentine carious lesions differ significantly and (2) CSH and SH used as alternatives to TMR are not adequate for estimating the mineral content of dentine.

Acknowledgments

The authors would like to thank The State of São Paulo Research Foundation (FAPESP) for the concession of a scholarship to the first author (process No. 2009/03581-9) and Prof. Dr. José Roberto Pereira Lauris for help with the statistical analysis. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' Contributions

A.C.M. conceived and designed the experiments; B.M.M., L.P.C., A.W. and H.Y. performed the experiments; A.C.M. and B.M.M. analyzed the data; A.C.M., B.M.M., A.W., M.A.R.B. and W.B. wrote the paper.

Disclosure Statement

The authors have no conflicts of interest to declare.

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